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The *Rhizobium leguminosarum* biovar *viciae* *nodO* gene can enable a *nodE* mutant of *Rhizobium leguminosarum* biovar *trifolii* to nodulate vetch

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Analysis of the nodulation characteristics of transposon-induced mutants of *Rhizobium leguminosarum* bv. *viciae* revealed that *nodO* and the closely-linked *rhi* genes contribute to nodulation of peas (*Pisum sativum*) and the vetch *Vicia hirsuta*. Although mutation of *nodO* alone had no significant effect on nodulation of either legume, a double mutant lacking both *nodO* and *nodE* nodulated both legumes very poorly. Similarly, a double mutant lacking *nodE* and either *rhiA* or *rhiB* nodulated peas less efficiently than a *nodE* mutant. Thus, although mutations affecting only the *rhi* genes normally have no observed effect on nodulation, these genes do appear to contribute to pea nodulation. When transferred to a wild-type strain of *Rhizobium leguminosarum* bv. *trifolii*, neither *nodO* nor the *rhi* gene region conferred pea or vetch nodulating ability. However, in a *nodE* mutant of *R. l.* bv. *trifolii*, *nodO* did confer a significant level of vetch nodulating ability, indicating that the secreted NodO protein can play a role in determining legume recognition by *R. l.* bv. *viciae*.

Keywords: *nodO*, nodulation, *Rhizobium leguminosarum*, *Vicia hirsuta*, *Pisum sativum*

INTRODUCTION

The rhizobial nodulation genes are the principal determinants of specificity in the recognition that occurs between rhizobia and their legume hosts. These genes are collectively known as *nod* or *nol* genes and the products of several of them are involved in the biosynthesis of lipo-oligosaccharide nodulation factors (Göttfert, 1993). These 'Nod' factors can induce complete or partial nodule organogenesis when added to the roots of some legumes even in the absence of bacteria (Truchet *et al.*, 1991; Spaink *et al.*, 1991) and purified lipo-oligosaccharide Nod factors can induce the formation of cytoskeletal changes to root cells similar to those structures that are made prior to the formation of mature infection threads (Van Brussel *et al.*, 1992). However, the development of normal infection threads requires the physical presence of bacteria expressing *nod* genes and the rhizobia grow along the infection threads to reach the newly-dividing plant cells that are destined to become the legume root nodule. It appears that, in addition to expressing the appropriate *nod* genes, infecting bacteria require an acidic exopolysaccharide, since mutants defective in its synthesis are unable to produce nodules containing rhizobia (see Leigh & Coplin, 1992).

Not all of the *nod* or *nol* genes are essential for nodulation of legumes. Mutation of certain *nod* genes results in a loss of ability to nodulate specific legume species within the normal cross-inoculation group of one rhizobial strain. For example, *nodV* and *nodW* mutants of *Bradyrhizobium japonicum* lose the ability to nodulate siratro (*Macroptilium atropurpureum*) but not soybean (Göttfert *et al.*, 1990). In other cases, mutation of *nod* genes can increase the range of legumes nodulated. Thus, a *nodE* mutant of *Rhizobium leguminosarum* bv. *trifolii* acquires the ability to nodulate peas (Djordjevic *et al.*, 1985).

Some mutations within specific *nod* genes have no discernible effect on nodulation of any of the legumes tested. In some strains of *R. l.* bv. *viciae*, mutations in *nodM*, *nodN*, *nodT* or *nodO* had no effect on nodulation (Surin & Downie, 1988, 1989; Surin *et al.*, 1990; Economou *et al.*, 1989). This may be due to gene duplication, as is the case for *nodM*, which encodes a glucosamine synthase, while a functional homologue is encoded by the housekeeping *glmS* gene (Marie *et al.*, 1992).

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Table 1. Bacterial strains and plasmids

Strain	Relevant characteristics	Source
A34	*R. l.* bv. *viciae* strain 8401/pRL1JI	Downie *et al.* (1983)
A42	Derivative of A34 carrying *nodE68*::Tn*5*	Downie *et al.* (1985)
A67	Derivative of A34 carrying *nodO93*::Tn*3lacZ*	This work
A69	Derivative of A34 deleted for *nodFELMNTO* and *rhiABCR*	Downie & Surin (1990)
A78	Derivative of A34 carrying *rhiB6*::Tn*3lacZ*	This work
A91	Derivative of A34 carrying *nodE68*::Tn*5* and *nodO93*::Tn*3lacZ*	This work
A93	Derivative of A34 carrying *nodE68*::Tn*5* and *rhiA5*::Tn*3lacZ*	This work
A95	Derivative of A34 carrying *nodE68*::Tn*5* and *rhiB6*::Tn*3lacZ*	This work
A99	Derivative of A34 carrying *rhiA5*::Tn*3lacZ*	This work
ANU843	*R. l.* bv. *trifolii*	Djordjevic *et al.* (1985)
ANU927	Derivative of ANU843 carrying *nodE*::Tn*5*	Djordjevic *et al.* (1985)
Plasmids		
pIJ1086	*nodLMNTO rhiABCR*	Downie *et al.* (1983)
pIJ1089	*nodABCIJFELMNTO rhiABCR*	Downie *et al.* (1983)
pIJ1332	Derivative of pIJ1085 carrying *nodE68*::Tn*5*	Downie *et al.* (1985)
pIJ1641	Derivative of pIJ1089 carrying *rhiB6*::Tn*3lacZ*	Economou *et al.* (1989)
pIJ1642	Derivative of pIJ1089 carrying *rhiA5*::Tn*3lacZ*	Economou *et al.* (1989)
pIJ1652	Derivative of pIJ1089 carrying *nodO93*::Tn*3lacZ*	Economou *et al.* (1989)
pIJ1685	Derivative of pIJ1086 carrying *nodO93*::Tn*3lacZ*	Downie & Surin (1990)
pIJ1750	Derivative of pIJ1086 carrying *rhiA9*::Tn*5 lacZ*	Cubo *et al.* (1992)
pIJ1788	*nodO* cloned on pLAFR1	Economou *et al.* (1990)

It is also evident that some genes which encode very different products may function synergistically. This was first noted with the *nodFE* and *nodO* genes (Downie & Surin, 1990); *nodF* and *nodE* encode proteins similar to the acyl carrier protein and condensing enzyme domains of the fatty acid synthase (Shearman *et al.*, 1986; Bibb *et al.*, 1989), and *nodE* is necessary for the synthesis of host-specific nodulation factors carrying the $C_{18:4}$ acyl group (Spaink *et al.*, 1991). In contrast, *nodO* encodes a secreted protein with the ability to form pores in membranes (Sutton *et al.*, 1993). A deletion mutant of *R. l.* bv. *viciae* lacking the *nodFELMNTO* genes (see Fig. 1) could not nodulate vetch, but could be partially complemented for nodulation by either a plasmid carrying *nodF* and *nodE* or cosmids carrying about 30 kb DNA including either *nodO* or the *nodLMNTO* gene region. Mutation of *nodO* abolished the ability of the cosmids to complement the deletion mutant (Downie & Surin, 1990). Thus, whereas mutation of *nodO* normally had little or no effect on nodulation of vetch, in the absence of the *nodFE* region, *nodO* was essential for the residual ($\approx 50\%$ of normal) nodulation seen with mutants lacking *nodE*. This result did not necessarily imply that *nodO* was the only gene on the cloned 30 kb region that contributed to the residual nodulation. Indeed, in a subsequent study, Cubo *et al.* (1992) demonstrated that mutations in a gene cluster close to *nodO* could reduce the efficiency with which the cosmid clone could complement nodulation in the deletion mutant. This gene cluster contains the *rhiA*, *rhiB*, *rhiC* and *rhiR* genes (see Fig. 1), which appear to be found in *R. l.* bv. *viciae* strains but not other rhizobia (Dibb *et al.*, 1984; Economou *et al.*, 1989). These genes were called *rhi* since they were expressed in the rhizosphere and not within nodules (Dibb *et al.*, 1984) and they are not under the same regulatory control as the *nod* genes (Economou *et al.*, 1989; Cubo *et al.*, 1992). Expression of the *rhiABC* genes depends on *rhiR*, which encodes a positively-acting transcriptional regulator with homology to known DNA-binding proteins (Cubo *et al.*, 1992). Interestingly, Economou *et al.* (1989) found that a mutation in *nodO* (then called *rolR*) resulted in a marked decrease in the amount of the *rhiA* gene product present in cells grown in the presence of hesperetin in order to induce *nod* gene expression. This indicated a possible link between NodO and the *rhi* genes although no biochemical function for any of the *rhiABC* gene products has been established.

In this work, we have subcloned *nodO* and shown it to be an important determinant of host specificity. In addition, we have constructed a series of double mutants containing a derivative of the transposon Tn*3* in *nodO* or the *rhiA* gene and the transposon Tn*5* in the *nodE* gene and measured the combined effects of these mutations on nodulation.

METHODS

Microbiological methods. *Rhizobium* strains are described in Table 1 and plasmids in Fig. 1 and/or Table 1. Plasmids were transferred by conjugation from *Escherichia coli* to the *R. leguminosarum* strains using the helper pRK2013 (Figurski & Helinski, 1979) and selecting on TY (complete) medium (Beringer, 1974) containing streptomycin (400 $\mu g\ ml^{-1}$) and

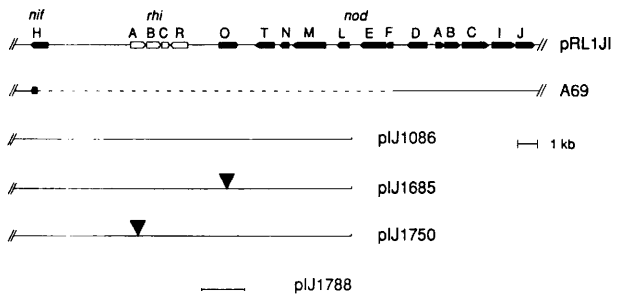

Fig. 1. Map of the *nod–rhi* gene region of *R. l.* bv. *viciae*. The locations, relative sizes and orientations of the *nod* (black arrows) and *rhi* (open arrows) genes on pRL1JI are indicated. The broken line indicates the deletion in strain A69. pIJ1086 and its derivatives pIJ1685 and pIJ1750, carrying the *nodO93*::Tn*3lacZ* and *rhiA9*::Tn*5lacZ* alleles (black triangles), respectively, are shown. The only *R. l.* bv. *viciae* gene on pIJ1788 is *nodO*.

tetracycline (5 μg ml^{-1}), or on Y (minimal) medium (Beringer, 1974) containing tetracycline (2 μg ml^{-1}). Selection for Tn*3*HoHo1 (hereafter referred to as Tn*3lacZ*) on pIJ1685 in *Rhizobium* was on the same media containing 50 μg carbenicillin ml^{-1}; 10 μg carbenicillin ml^{-1} was used to select for Tn*3lacZ* alleles on pRL1JI.

It was possible to construct double mutants since *rhiA*, *rhiB* and *nodO* mutations had previously been isolated using the transposon Tn*3lacZ* (Economou *et al.*, 1989; Cubo *et al.*, 1992) and a *nodE* mutation had been isolated using Tn*5* (Downie *et al.*, 1985). Strains A67, A78 and A99 were made by transferring pIJ1652, pIJ1641 and pIJ1642 (Economou *et al.*, 1989) into A34 and transferring the *nodO93*::Tn*3lacZ*, *rhiB6*::Tn*3lacZ* and *rhiA5*::Tn*3lacZ* alleles, respectively, onto the symbiotic plasmid pRL1JI by homologous recombination as described by Ruvkun & Ausubel (1981), selecting for the Tn*3lacZ* alleles using carbenicillin. Plasmid pIJ1332, carrying the *nodE68*::Tn*5* allele, was then introduced into strains A67, A78 and A99 and the *nodE68*::Tn*5* allele on pIJ1332 transferred onto the symbiotic plasmid pRL1JI by homologous recombination, selecting for the Tn*5* allele with kanamycin. The recombinants were screened for sensitivity to tetracycline (to ensure loss of the vector) and shown to retain the Tn*3lacZ* alleles by confirming that the expected levels of expression of β-galactosidase were retained. *Eco*RI-digested DNA from the double mutant strains A91, A93 and A95 was then confirmed to have the appropriate pattern of bands following hybridization with labelled pIJ1089, which contains DNA spanning this region. This confirmed that no rearrangements had occurred within the *nod–rhi* gene region following recombination and that both transposons were in the appropriate locations.

Plant tests. Nodulation tests with the vetch *Vicia hirsuta* were carried out on agar slopes as described by Knight *et al.* (1986), using a minimum of 20 plants per assay. Pea nodulation tests were done with *Pisum sativum* variety Wisconsin Perfection as described previously (Knight *et al.*, 1986), using a minimum of 12 plants per assay. The results of tests on both species are expressed in Figs 2–5 as mean values with bars representing ±SE. In those cases where low levels of nodulation were observed, bacteria were isolated from six surface-sterilized nodules and reinoculated onto fresh plants. In each case this resulted in a similar delayed or low level of nodulation, indicating that reversion was not a significant problem. Measurements of acetylene reduction were made as described by Knight *et al.* (1986).

RESULTS

Nodulation phenotypes of double mutants

The nodulation efficiency of double mutants lacking *nodE* and *nodO* (strain A91) or *nodE* and *rhiA* (strain A93) or *nodE* and *rhiB* (strain A95) was compared with mutants carrying single mutations (Fig. 2). Mutation of *nodO* alone (strain A67) had no significant effect on nodulation of peas or vetch and mutation of *nodE* (strain A42) lowered the nodulation efficiency by about 30–40%. However, the

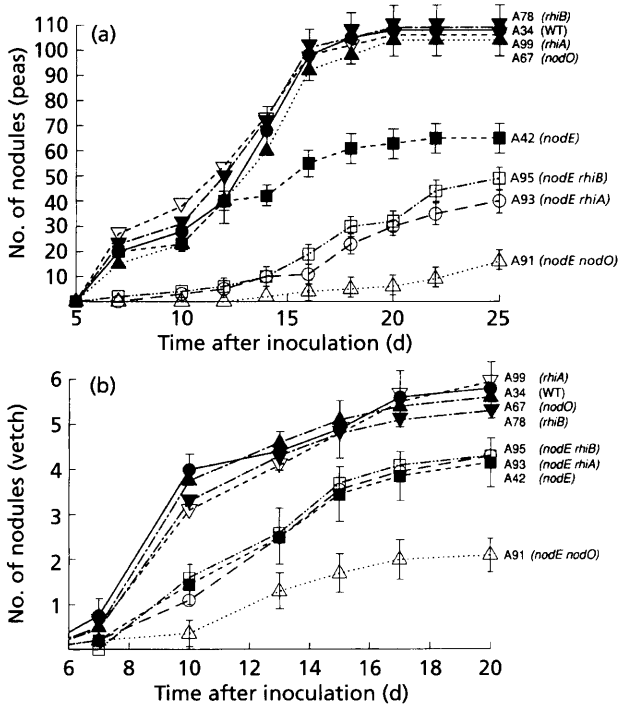

Fig. 2. Nodulation of peas and vetch (*V. hirsuta*) by double mutants of *R. l.* bv. *viciae*. The mean numbers of nodules formed on peas (a) and vetch (b) by various strains of *R. l.* bv. *viciae* are shown: ●, A34; ■, A42; ▲, A67; ▼, A78; △, A91; ○, A93; □, A95; and ▽, A99. For simplicity of presentation only selected (but typical) standard errors are shown. In (a) the standard errors shown correspond to those found with A34, A42, A95, A93 and A91; these values are similar to those found using the other strains, which indicate that there is no significant difference among A78, A34, A99 and A67 or between A95 and A93. In (b) the standard errors shown correspond to those found with A34, A42 and A91. Similar values were found with the other strains, indicating that the nodulation profiles of A34, A78, A99 and A67 are not significantly different from each other but are different from the others (particularly 10–18 d after incubation). Similarly A93, A95 and A42 do not differ significantly from each other but are different from A91 (particularly 18–25 d after incubation). The nodules formed were all pink and could reduce acetylene to ethylene, indicating that they contained nitrogen-fixing bacteroids.

double mutant (A91) carrying transposons in both *nodO* and *nodE* poorly nodulated both peas (Fig. 2a) and vetch (Fig. 2b), indicating that *nodO* and *nodE* have additive effects on nodulation. It had been anticipated in the light of previous work (Downie & Surin, 1990) that the double mutant might be unable to form nodules and the low residual level of nodulation seen here had not been expected.

The combination of the *rhiA5*::Tn*3lacZ* or the *rhiB6*::Tn*3lacZ* allele together with the *nodE68*::Tn*5* mutation (strains A93 and A95, respectively) also reduced the level of pea nodulation (Fig. 2a) compared with the effects of individual mutations in *rhiA* or *rhiB* (A78 and A99) or *nodE* (A42). The effect of the double mutation of *rhiA* and *nodE* (A93) or *rhiB* and *nodE* (A95) on nodulation was not as strong as that seen with mutation of both *nodO* and *nodE* (A91). These effects are consistent with the *rhi* genes playing an ancillary role in pea nodulation as concluded previously (Cubo *et al.*, 1992). On vetch, however, mutation of *rhiA* (A93) or *rhiB* (A95) in addition to mutation of *nodE* had no effect above that of mutation of *nodE* alone (A42) (Fig. 2b). In contrast, Cubo *et al.* (1992) noted that, in the absence of *nodE*, mutation of *rhi* genes did have an effect on nodulation of vetch. However, in those experiments the *nodL* gene product was also absent; this difference may account for the apparent discrepancy with the results seen here.

nodO and *rhi* genes have additive effects on nodulation

It has not been established whether the *nodO* gene alone can compensate for the nodulation defect of a strain lacking the *nodFE* gene region, or whether other genes (such as the *rhi* genes) are necessary to allow *nodO* complementation to be expressed (e.g. the *rhi* genes could be involved in an activation of NodO). This was addressed by transferring the subcloned *nodO* gene on pIJ1788 into the deletion mutant A69 (carrying *nodABCDIJ* but lacking *nodFELMNTO* and *rhiABCR*). The transconjugant induced a low level of nodulation on vetch, a property that A69 lacks (Downie & Surin, 1990) (Fig. 3). The level of nodulation was similar to that seen with A69 containing pIJ1750, a derivative of pIJ1086 (*nodLMNTO*, *rhiABCR*) carrying the *rhiA9*::Tn*5lacZ* allele. These observations indicate that, in the absence of the *rhi* gene region, *nodO* is less efficient at restoring nodulation to the deletion mutant A69. The nodules formed contained many bacteria as judged by light microscopy of fixed nodule sections (data not shown).

nodO can confer vetch nodulating ability on a *nodE* mutant of *R. l.* bv. *trifolii*

The observation that *nodO* could allow strain A69 to nodulate vetch led us to investigate the possibility that *nodO* might confer some level of host specificity in nodulation. *nodO* on pIJ1788 was transferred into several strains of *R. l.* bv. *trifolii* and *R. l.* bv. *phaseoli*. In some cases the different *R. l.* bv. *trifolii* transconjugants induced

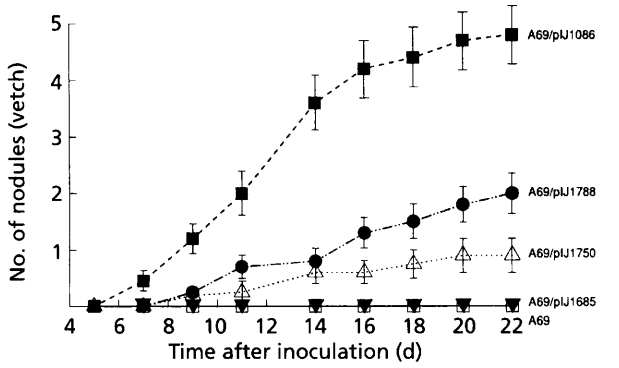

Fig. 3. Nodulation of vetch by *R. l.* bv. *viciae* strain A69 (▼), and derivatives of it carrying pIJ1086 (■), pIJ1685 (□), pIJ1750 (△) or pIJ1788 (●). pIJ1685 is a derivative of pIJ1086 carrying *nodO93*::Tn*3lacZ*; pIJ1750 is a derivative of pIJ1086 carrying *rhiA9*::Tn*5lacZ*; pIJ1788 carries the cloned *nodO* gene.

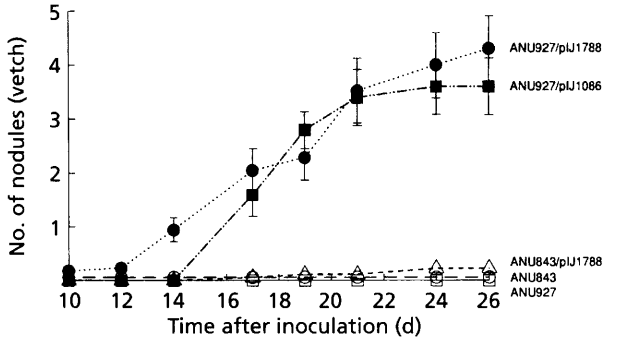

Fig. 4. Nodulation of vetch by *R. l.* bv. *trifolii* strains ANU843 (○), ANU843/pIJ1788 (△), ANU927, either with or without pIJ1685 (□), ANU927/pIJ1086 (■), ANU927/pIJ1788 (●).

very low levels of nodulation on vetch but this was erratic. For example, when pIJ1788 was introduced into the well-characterized strain of *R. l.* bv. *trifolii* ANU843, only about 15% of the vetch plants inoculated formed one or two nodules after 21 d (Fig. 4). With the *R. l.* bv. *phaseoli* transconjugants, no nodulation of vetch was seen.

Djordjevic *et al.* (1985) reported that a *nodE* mutant of strain ANU843 could nodulate peas and we have confirmed this observation with its *nodE* mutant, ANU927 (Fig. 5). Surprisingly strain ANU927 did not nodulate vetch (Fig. 4). We were unable to detect a *nodO* homologue in strain ANU843 either by DNA hybridization or using NodO antiserum (Scheu *et al.*, 1992). However, when *nodO* on pIJ1788 was transferred into strain ANU927, it acquired the ability to nodulate vetch (Fig. 4). A similar level of nodulation was seen with strain ANU927 containing plasmid pIJ1086. This clearly demonstrates an involvement of *nodO* in the host-specific nodulation of vetch. The nodules formed on vetch by strain ANU927/pIJ1788 did contain large numbers of bacteroid

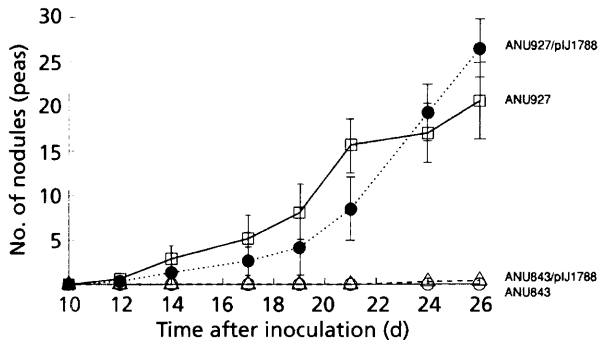

Fig. 5. Nodulation of pea by *R. l.* bv. *trifolii* strains ANU843 (○), ANU843/pIJ1788 (△), ANU927 (□) and ANU927/pIJ1788 (●).

forms as judged by light microscopy of nodule sections (data not shown), indicating that *nodO* stimulated the infection process. In parallel experiments with peas, plasmid pIJ1788 did not increase the level of nodulation seen with strain ANU927 (Fig. 5).

To determine if the *rhiABCR* and the *nodLMNT* genes contribute any host specificity in nodulation, pIJ1685 (the derivative of pIJ1086 carrying *nodO93*::Tn*3lacZ*) was transferred into strain ANU927 and the transconjugants were tested for pea and vetch nodulation. There was no nodulation of vetch, indicating that the *rhiABCR* and *nodLMNT* genes present on pIJ1685 do not extend the host-range of nodulation in this system.

DISCUSSION

Whereas mutations in the R. *l.* bv. *viciae nodA*, *nodB*, *nodC* and *nodD* genes block nodulation, it is clearly difficult to uncover the relative contributions of some of the *nod* and other genes (such as *rhi* genes) to the overall efficiency of nodulation. One clear test is to transfer the appropriate genes into another biovar and check for extension or alteration of host range. This strategy previously revealed that the *nodD* and *nodFE* genes from R. *l.* bv. *viciae* determined specificity in R. *l.* bv. *trifolii* (Spaink *et al.*, 1987, 1989; Surin & Downie, 1989). In this work we have demonstrated that *nodO* can also act as a determinant of vetch nodulation specificity in a *nodE* mutant of R. *l.* bv. *trifolii*.

The *nodO* gene encodes a secreted protein (de Maagd *et al.*, 1989; Economou *et al.*, 1990) which binds Ca^{2+} (Economou *et al.*, 1990). In contrast, several of the other *nod* gene products are involved in the synthesis of lipo-oligosaccharide Nod factors that are key determinants of specificity (Göttfert, 1993). In particular, the *nodE* gene product is involved in the formation of the novel $C_{18.4}$ acyl group on the R. *l.* bv. *viciae* host-specific Nod factors and in the absence of *nodE* only $C_{18:1}$-containing Nod factors are made (Spaink *et al.*, 1991). The synergistic functions of NodO with the other *nod* gene products are unlikely to occur at the level of modification of the Nod factors, because these factors are identical if made in strains carrying or lacking *nodO* (Spaink *et al.*, 1991). Instead, NodO may contribute to nodulation efficiency by forming ion channels in plant plasma membranes (Sutton *et al.*, 1994). Interestingly, Ehrhardt *et al.* (1992) found that the *Rhizobium meliloti* lipo-oligosaccharide nodulation factor induced a depolarization of the plasma membrane of alfalfa root hair cells. Therefore NodO might increase or amplify the effect of the lipo-oligosaccharide Nod factor, but it is not clear how this could contribute to nodulation specificity. If the inappropriate Nod factors (or ratio of Nod factors) are made by the *nodE* mutant of R. *l.* bv. *trifolii*, perhaps only a very weak root hair depolarization is achieved and NodO can in some way amplify this effect. Alternatively, NodO might facilitate the uptake of Nod factors.

One way of looking at the contribution of the various genes involved in nodulation is to consider that nodulation tests may not necessarily be a good measure of the potential nodulation proficiency of a given rhizobial strain. By this we mean that if the nominal nodulation proficiency were reduced by, for example, 50%, the number of nodules formed on a test legume might be normal because the plant can compensate for lower proficiency by allowing more infections to proceed to nodules. There are clear data that support the idea that the plant controls the final numbers of nodules formed (Caetano-Anolles & Gresshoff, 1991) and that only a small proportion of infection events lead to nodule formation (Vasse *et al.*, 1993). Thus, in the absence of some *nod* genes, no effect might be seen on nodulation efficiency. However, when the level of nodulation is substantially reduced (e.g. by the loss of *nodE*), bacterially-determined nodulation signals could become the limiting factor and the legume may no longer be able to compensate for the inefficiency in nodulation. Under these conditions it would then become possible to observe effects on nodulation of genes that otherwise appear to be unimportant. If this were the case, it could explain why the loss of *nodO* or *rhi* gene expression only affects the level of nodulation of peas when *nodE* is also absent. Thus, it is possible that NodO may stimulate nodulation proficiency, in either the presence or absence of *nodE*, but this stimulation is actually only seen when the nodulation efficiency is very severely reduced, for example when only $C_{18:1}$ acylated Nod factors are made as seen when *nodE* is absent (Spaink *et al.*, 1991). It follows that there might be other, as yet unidentified, genes that could contribute to nodulation. However, the observations that *nodO* does not stimulate nodulation of peas by the *nodE* mutant of R. *l.* bv. *trifolii* and that the *nodE rhiA* double mutant nodulates vetch at the same efficiency as the *nodE* mutant, indicate that an incremental loss of nodulation efficiency is not the whole explanation. It is possible that other components such as activation of plant defences may also be important in the infection of legumes (Vasse *et al.*, 1993). The precise mechanisms by which the *nodO* and *rhi* gene products stimulate nodulation have yet to be established but they appear to be rather different from any components yet identified in other rhizobial strains.

It seems that a key role for NodO may be to stimulate

infection thread formation and this may be particularly important in mutants lacking *nodE* which make inappropriate lipo-oligosaccharide nodulation factors. If NodO does function by forming pores in the plasma membrane, its most likely site of action would be at the tip of the infection thread where the plant cell wall is being synthesized and is much less likely to prevent the access of NodO to the plasma membrane. This offers the interesting possibility of an ion flux across the root-hair cell membranes in a localized region and this could be important for the cytoskeletal changes that are required for maintenance of infection thread growth.

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